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(54) Title: MICROBIAL 11 α -HYDROXYLATION OF STEROIDS (57) Abstract The invention relates to a microbial method of in vitro transformation of a steroid into its corresponding 11 α -hydroxy analogue using oxygen and a microorganism selected from <i>Aspergillus ochraceus</i> , <i>Aspergillus niger</i> , <i>Rhizopus stolonifer</i> , <i>Rhizopus nigricans</i> , <i>Rhizopus arrhizus</i> , and strains of <i>Pestalotia</i> , characterized in that a steroid having a purity of less than 97 % is used.		

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MICROBIAL 11 α -HYDROXYLATION OF STEROIDS

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The present invention relates to a microbial method of 11 α -hydroxylation of steroids.

Microbial 11 α -hydroxylation of steroids is a well known process, in vivo as well as in vitro. For instance 11 α -hydroxylation of progesterone by cell-free preparations of *Aspergillus*
10 *ochraceus* has been reported by Shibahara et al., Biochim. Biophys. Acta, 202 (1970), 172-179. It has also been known that microbial 11 α -hydroxylation reactions of steroids are unpredictable, and invariably lead to incomplete transformations. Typically conversion degrees of 80-85% are obtained. For industrial applications it is however of importance to obtain high predictable conversion rates, which preferably lead to higher than 95% yields of 11 α -
15 hydroxylated steroids. Mathematical models in the optimization of such fermentation processes are discussed by Deshayes et al., Bull. Soc. Chim. Fr., (1980), II 24-34. For example, in the 11 α -hydroxylation of canrenone, Deshayes disclosed that under optimum conditions better than 95% yields could be attained when *Aspergillus ochraceus* was used in a medium containing i.a. 10 g/l of glucose in a matrix of malt-extract and trypticase. Under these conditions up to 1.5 g/l
20 of canrenone could be transformed, which was considered to be an improvement in the art, for instance as disclosed by Blunt et al., J. Chem. Soc., 6 (1971), 1136, who were not able to obtain more than 90% yield of 11 α -hydroxylated canrenone using at the most 0.5 g/l of substrate.

25 Since it can be reasoned that contaminations will have a detrimental influence on the microbial process, it can be expected that further optimization could be obtained by increasing the purity of the starting materials. Surprisingly however, it has now been found that a further improvement is obtained by using less than pure substrate, in particular by using a substrate having a purity of less than 97%. The invention therefore relates to a microbial method of in
30 vitro transformation of a steroid into its corresponding 11 α -hydroxy analogue using oxygen and a micro-organism selected from *Aspergillus ochraceus*, *Aspergillus niger*, *Rhizopus stolonifer*, *Rhizopus nigricans*, *Rhizopus arrhizus*, and strains of *Pestalotia*, characterized in that a steroid having a purity of less than 97% is used.

Preferably the micro-organism is *Aspergillus ochraceus*. The purity of the steroid is preferably more than 90%. More preferably the purity of the steroid is between 90 and 95 %. The present method using impure substrates affords microbial transformations at substrate concentrations, which are substantially greater than the maximum concentrations as disclosed by Blunt or Deshayes.

The present microbial method can be used with steroids having an unsubstituted 11-position. Preferred examples are estr-4-ene-3,17-dione and canrenone.

The surprising effect of impurities on the conversion degree is illustrated in the following tables:

Table I

Conversion of estr-4-ene-3,17-dione by <i>A. ochraceus</i>		
purity (%)	substrate concentration (g/l)	conversion degree (%)
99	15	78
99	10	83
98	25	85
94*	15	91
94*	15	91
94*	15	94
93	25	97
92	10	98

* various natural impurities added to pure substrate

Table II

Conversion of canrenone by <i>A. ochraceus</i>		
purity (%)	substrate concentration (g/l)	conversion degree (%)
100	5	74
100	10	78
100	20	73
100	35	72
96	10	98
94	15	96
96	22	96
95	22	95

The invention is further illustrated by the following examples.

EXAMPLES

Example 1

A shake flask containing a mineral growth medium with glucose was inoculated with
5 spores of *A. ochraceus* and placed on a reciprocal shaker at 28 °C for 15 h. A stirred fermentor
containing 5 l of medium was subsequently inoculated with 250 ml of germinated spore
suspension. The used medium contained a glucose/yeast extract medium (glucose 40 g/l, yeast
extract 10 g/l, pH 5.0). The culture conditions were as follows: stirrer speed 750 rpm, airflow
0.2 l/l/m, temp 28 °C. Foaming was measured with an antifoam electrode and controlled by
10 automatic addition of a silicon based antifoam agent. When the culture had a biomass
concentration of at least 2 g/l a small portion of estr-4-ene-3,17-dione (1 g/l) was added to
induce the synthesis of the hydroxylation enzymes. Three hours later higher concentrations of
steroid were added to reach the desired concentrations as given in Table I. The steroid trans-
formation was stopped when repeatedly no increase in conversion was observed.

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Example 2

In a shake flask the same culture as described in Example 1 was prepared. When the
culture had a biomass concentration of at least 4 g/l the canrenone was added at once to reach
the desired concentrations as given in Table II. The steroid transformation was stopped when
20 repeatedly no increase in conversion was observed.

CLAIMS

1. A microbial method of in vitro transformation of a steroid into its corresponding 11 α -hydroxy analogue using oxygen and a micro-organism selected from *Aspergillus ochraceus*,
5 *Aspergillus niger*, *Rhizopus stolonifer*, *Rhizopus nigricans*, *Rhizopus arrhizus*, and strains of *Pestalotia*, characterized in that a steroid having a purity of less than 97% is used.
2. The method according to claim 1, wherein the micro-organism is *Aspergillus ochraceus*.
10
3. The method according to claim 1 or 2, wherein the purity of the steroid is between 90 and 95%.
4. The method according to any one of claims 1-3, wherein a steroid concentration
15 greater than 0.5 g/l is used.
5. The method according to any one of claims 1-4, wherein the steroid is selected from estr-4-ene-3,17-dione and canrenone.

INTERNATIONAL SEARCH REPORT

International Application No

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A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12P33/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 3 294 646 A (SMITH, LELAND LEROY ET AL) 27 December 1966 see claims	1,2,5
Y	--- BULL. SOC. CHIM. FR. (1980), (1-2, PT. 2), 24-34 CODEN: BSCFAS; ISSN: 0037-8968, 1980, XP002029426 DESHAYES, CHRISTIAN M. P.: "Use of mathematical models for optimization in fermentation. Applications to transformations by microorganisms" cited in the application see page 31 --- -/--	1,2,5

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	FR 1 091 743 A (PFIZER) 14 April 1955 see claims ---	1
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International Application No

PCT/EP 96/05729

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